International Journal of Pharmacology and Toxicology, *1 (2) (2013) 64-81*
©Science Publishing Corporation
www.sciencepubco.com/index.php/IJPT

Antibacterial activity of novel β-Lactam moieties on *α-Hemolytic Streptococcus*

Swaroop G*, Bernard T Golding, Ulrich Baisch, Gary Kirk, Ashwini Karakaraddi, Prathibha K S

MS (Medicinal Chemistry), Newcastle University, United Kingdom & PhD scholar (Medical Oncology), International University for Complementary Medicine, Colombo
Senior Research Investigator, Newcastle University, United Kingdom
Head of Chemical Crystallography, Newcastle University, United Kingdom
Analytical Chemist, GlaxoSmithKline Pharmaceuticals, United Kingdom
Head of the Department of Biotechnology, K.L.E's S. Nijalingappa College, Bangalore, India.
Lecturer, Department of Biotechnology, K.L.E's S. Nijalingappa College, Bangalore, India.
**Corresponding author E-mail: simhavahini23@gmail.com*

Abstract

Synthesis of novel β-lactam antibiotic drug moieties deals with the bicyclic compound, aim to improve the good antibacterial activity. The β-lactam antibiotics moieties were synthesised via coupling of a range of acids such acetyl salicylic acid; benzoic acid; phenoxyacetic acid to the β-lactam backbone moiety of 6-aminopenicillanic acid (6-APA). Initially various coupling methods were investigated and optimized for Model amide reactions (i) NHS/DCC, (ii) EDC (iii) Mixed anhydride reactions respectively, followed by deprotection of penicillin by BOC derivatives. Study of Kinetics on Benzyl Penicillin (BP) was carried out to detect the rate of hydrolysis of the drug. The synthesized antibacterial drugs were administered on α-Hemolytic Streptococcus bacterial species for the bioassay studies through zone of inhibition method.

Keywords: *Antibiotic (Ab), 6-Amino Penicillin Acid, Antibacterial activity, Dichloromethane (DCM), [N-(3 dimethylaminopropyl)-N'-ethyl carbodiimide HCl] (EDC), N, N Dimethylformamide (DMF), N N' Dicyclohexylcarbodiimide (DCC), N-acetyl mumaric acid (NAM), N-acetyl glucosamine (NAG), α-hemolytic Streptococcus, Streptomycin, Tetracycline.*

1 Introduction

There is a continuous need for new antibiotics to overcome antibiotic-resistant strains of organisms. However the antibiotic development has been very slow with only four new classes of antibiotics being introduced into pharmaceutical market [1]. The inevitable rise of resistance will erode the utility of today's antibiotics. To date most of the pharmaceutical research has concentrated on improving the pharmacokinetics and pharmacodynamic properties of the drug [1].

Penicillin contains a highly unstable bicyclic system consisting of 4 membered β-lactam rings fused to a five membered thiazolidine ring. The skeleton of the molecule is derived from the amino acids cysteine and valine [2].

The bicyclic system of penicillin goes through a large angle and torsional strains. Acid-catalysed ring opening relieves these rings by breaking open the more highly strained four-membered ring. The β-lactam of penicillin is usually more reactive toward nucleophiles than are normal amides. Substituent effects on both the acyl and amine portions of the β-lactam should be considered. For example, carbon- β-lactam nitrogen bond fission in penicillin's involves the removal of a much more weakly basic amine than that normally found in amides. A simple way to determine the reactivity of the β-lactam antibiotics is to examine their rates of hydrolysis [2].

The remarkable four-membered β-lactam ring of penicillin, which was so decisively revealed by Hodgkin's crystallographic analysis (1945), also turned out to be the motif that was responsible for the lethal action of the drug against bacteria. This activity was found to be related to the conformation adopted by penicillin, where in the fused 4, 5-ring system enforces an orthogonal alignment of the nitrogen lone pair and the carbonyl π-bond such that the resonance stabilization exhibited by traditional amides cannot be attained in this case. This feature, in combination with the intrinsic strain of the four-membered ring, creates a situation where the carbonyl functionality of the β-lactam ring acts as a highly effective acylating agent due to its particularly strong electrophilic reactivity. Thus, it is now known that penicillin irreversibly acylates the bacterial transpeptidase enzyme responsible for the cross-linking reaction which

unites the terminal glycine residue of a pentaglycine strand with the D-ala residue of a neighbouring pentapeptide, in a crucial step during the construction of bacterial cell walls. The acylation process deactivates this crosslinking enzyme, thereby compromising the integrity of the bacterial cell wall, resulting in rapid cell death. Unlike bacteria, only a phospholipid membrane surrounds mammalian cells, so transpeptidase inhibition is completely selective for bacterial cells. It has been shown that the penicillin molecule adopts an overall conformation that is very similar to the D-ala-D-ala residue of the substrate involved in this chain elongation process, thus it gains ready access to the active site of the enzyme where it reacts to disable its host [3].

Penicillin is in epitome an acylated cyclic dipeptide containing the amino acids L-cysteine and D-valine. It can be viewed as an analogue of the acylated D-alanyl-D-alanine in the linear glycopeptide. The occurrence of D-iso-glutamine in the cell wall and in the nascent glycopeptide units makes it unlikely that penicillin with its free carboxyl group is an analogue of L-alanyl-D-glutamic acid in the peptide chain and no evidence has been presented to support an earlier idea that penicillin is a structural analogue of NAM. In fact, the terminal D-alanine contains the only free carboxyl group in the nascent glycopeptide which might resemble that in penicillin. The atoms of the peptide chain in penicillin are fixed in position by the ring system. One of the possible conformations of the peptide chain of D-alanyl-D-alanine is almost identical to that of penicillin. The methyl group of the D-alanyl residue has a proton as its analogue in the penicillin molecule [4].

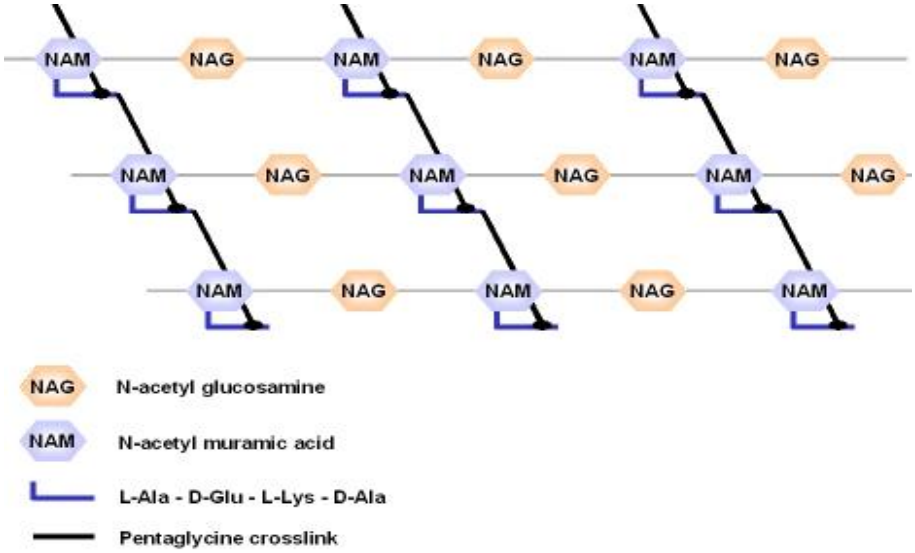


Fig. 1: Biochemistry of bacterial cell wall [5]

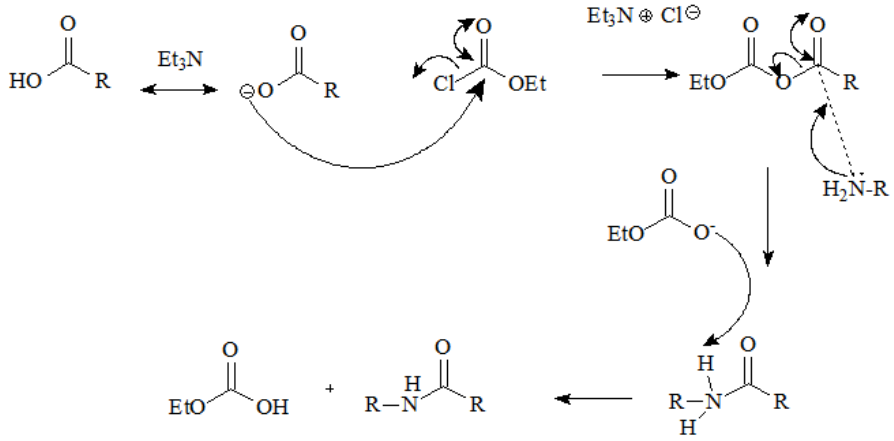
Discovery of 6-APA: The novel penicillin was synthesized using p-amino benzyls as the starting material. The modifications were carried at the p-amino group in the β-lactam scaffold. The discovery of 6-Aminopenicillanic acid (6-APA) made a range of discovery of β-lactam antibiotics as an antibacterial agent [6]. Medicinal chemists are carrying out remarkable work in the field of β-lactam chemistry, as there is a need for the potent and effective β-lactam antibiotic drugs thereby designing new functionalised 2-azetidinones. These are also used as the synthons in the preparation of variety of heterocyclic compounds.

2 Materials and Methods

2.1 Model amide reactions carried to select the appropriate method for penicillin synthesis:

The starting materials were selected with similar pKa value and nucleophilicity. In the preparation of model amides/anilines the following methods were examined through DCC method and mixed anhydride method.

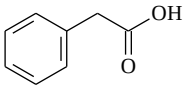
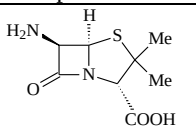
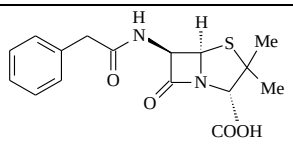
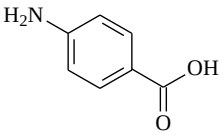
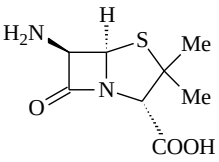
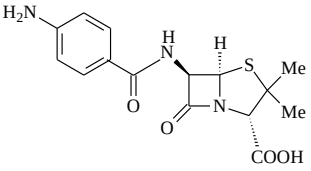
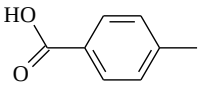
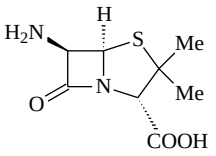
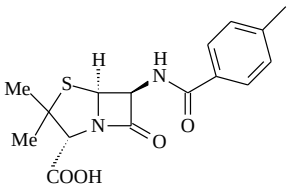
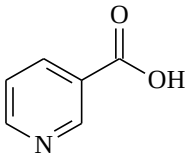
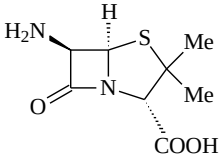
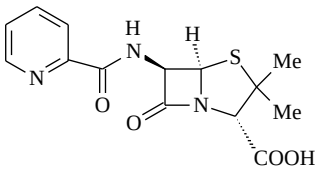
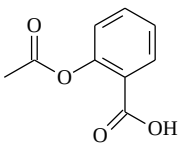
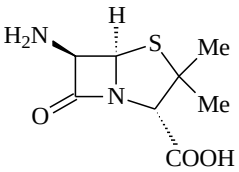
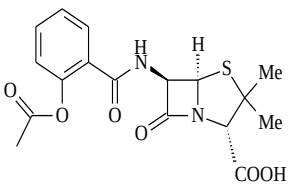
Reaction Mechanisms:

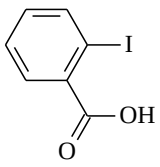
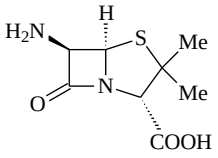
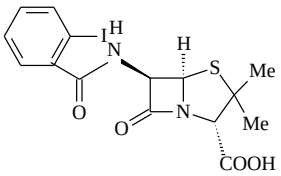
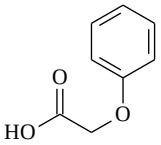
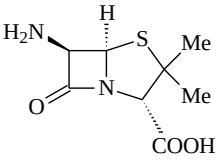
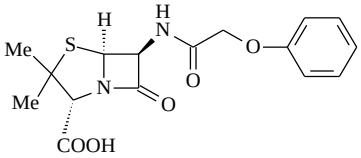
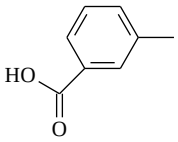
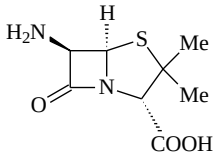
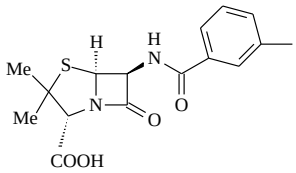
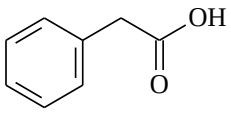
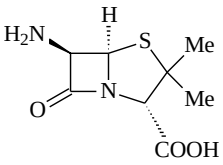
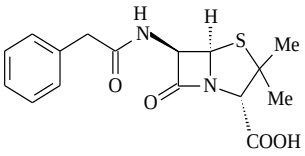


Scheme. 1: (Reaction mechanism for mixed anhydride method)

The carboxylic acid derivative reacts with ethylchloroformate in presence of triethylamine as a base and Dry Dichloromethane (DCM) to give the triethylamine salt ions resulting in the formation of anhydride complex. The nucleophilic N of the amine attacks the carbonyl carbon of the carboxylic acid forming the amide and acid [8].

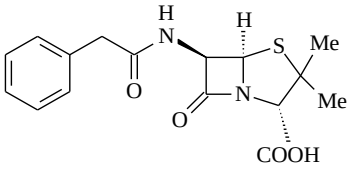
Table 1: Scheme for the preparation of β-lactam antibiotic moieties

Sl. No	Method used	Starting material	6-Aminopenicillanic acid	Product
1.	Mixed Anhydride	 **Phenylacetic acid**	 **6-APA**	 **Beta-lactam Antibiotic 1**
3.	Mixed Anhydride	 **4-Aminobenzoic acid**	 **6-APA**	 **Beta-lactam Antibiotic 3**
5.	Mixed Anhydride	 **p-Toluic acid**	 **6-APA**	 **Beta-lactam Antibiotic 5**
6.	Mixed Anhydride	 **Nicotinic acid**	 **6-APA**	 **Beta-lactam Antibiotic - 6**
7.	Mixed Anhydride	 **Aspirin (Acetyl Salicylic acid)**	 **6-APA**	 **Beta-lactam Antibiotic - 7**

11.	Mixed Anhydride	 **2-Iodobenzoic acid**	 **6-APA**	 **Beta-lactam Antibiotic - 11**
12.	Mixed Anhydride	 **Phenoxyacetic acid**	 **6-APA**	 **Beta-lactam Antibiotic - 12**
14.	Mixed Anhydride	 **m-Toluic acid**	 **6-APA**	 **Beta-lactam Antibiotic - 14**
16.	Mixed Anhydride	 **Phenylacetic acid**	 **6-APA**	 **Beta-lactam Antibiotic - 16**

2.2 Synthesis of β-lactam antibiotics

2.2.1 Preparation of Phenyl acetic acid to 6-Aminopenicillanic acid



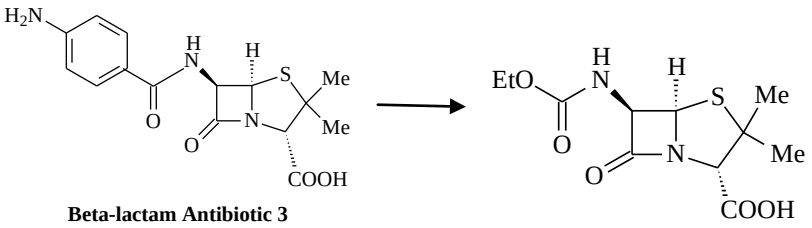
Beta-lactam Antibiotic 1

$C_{15}H_{18}O_4N_2S$

Phenylacetic acid (0.55 g, 4 mmol), triethylamine (0.55 mL, 4 mmol) was dissolved in dry DMF (5 mL). The reaction mixture is cooled to -10º C in ice/salt bath and ethyl chloroformate (0.38 mL, 4 mmol) was added drop wise to the reaction mixture was left to stir for 20 min followed by the addition of dry ice cold acetone (5 mL). A chilled solution was prepared previous day containing 6-APA (0.87g, 4 mmol), triethylamine (0.40 mL, 4 mmol) in water (10 mL) was added and the reaction is left to stir for 90 min and is allowed to return to room temperature.

The reaction mixture is quenched with the addition of water (5 mL) and the pH is adjusted to 7. The organic layer is washed with ethyl acetate (10 mL) and acidified to pH 2 and immediately extracted by ethyl acetate (3 x 10 mL). The ether layer is washed with water (2 x 10 mL) and extracted with 1M potassium bicarbonate solution which was then washed with ethyl acetate (2 x 10 mL) and freeze dried to give potassium salt of Penicillin. And the resulted mixture is evaporated under vacuum pressure. The obtained product was dissolved in methanol to remove the impurities with some amount of dry ether and it is evaporated again to get the pure form of penicillin derivative [8].

2.2.2 Attempt for the preparation of 4-Aminobenzoic acid to 6-Aminopenicillanic acid (Ethoxy Penicillin)

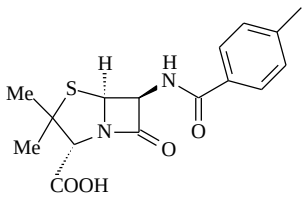


Beta-lactam Antibiotic 3

$C_{15}H_{17}O_4N_3S$ **$C_{11}H_{15}O_5N_2S$**

4 Aminobenzoic acid (0.274 g, 2 mmol), triethylamine (0.20 mL, 2 mmol) was dissolved in dry DMF (2.5 mL). The reaction mixture is cooled to -10ºC in ice/salt bath and ethyl chloroformate (0.217 mL, 2 mmol) was added drop wise to the reaction mixture was left to stir for 20 min followed by the addition of dry ice cold acetone (2.5 mL). A chilled solution was prepared previous day containing 6-APA (0.433 g, 2 mmol), triethylamine (0.20 mL, 4 mmol) in water (5 mL) was added and the reaction is left to stir for 90 min and is allowed to return to room temperature [8]. The reaction mixture is worked up as in procedure 2.2.1.

2.2.3 Preparation of p-Toluic acid to 6-Aminopenicillanic acid

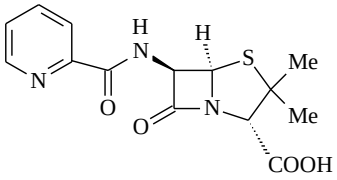


Beta-lactam Antibiotic 5

$C_{16}H_{18}O_4N_2S$

p-Toluic acid (0.273 g, 2mmol), triethylamine (0.20 mL, 2mmol) was dissolved in dry DMF (2.5 mL). The reaction mixture is cooled to -10ºC in ice/salt bath and ethyl chloroform ate (0.217 mL, 2mmol) was added drop wise to the reaction mixture was left to stir for 20 min followed by the addition of dry ice cold acetone (2.5 mL). A chilled solution was prepared previous day containing 6-APA (0.433 g, 2 mmol), triethylamine (0.20 mL, 2 mmol) in water (5 mL) was added and the reaction is left to stir for 90 min and is allowed to return to room temperature. The reaction mixture is worked up as in procedure 2.2.1

2.2.4 Preparation of coupling of Nicotinic acid to 6-Aminopenicillanic acid



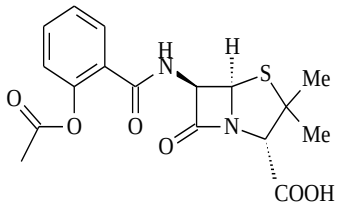
Beta-lactam Antibiotic - 6

$C_{14}H_{15}O_4N_3S$

Nicotinic acid (0.246g, 2mmol), triethylamine (0.20 mL, 2mmol) was dissolved in dry DMF (2.5 mL). The reaction mixture is cooled to -10ºC in ice/salt bath and ethyl chloroformate (0.217 mL, 2mmol) was added drop wise to the reaction mixture was left to stir for 20 min followed by the addition of dry ice cold acetone (2.5 mL).

A chilled solution was prepared previous day containing 6-APA (0.433 g, 2 mmol), triethylamine (0.20 mL, 2 mmol) in water (5 mL) was added and the reaction is left to stir for 90 min and is allowed to return to room temperature. The reaction mixture is worked up as in procedure 2.2.1.

2.2.5 Preparation of coupling of Acetyl Salicylic acid (Aspirin) to 6-Aminopenicillanic acid

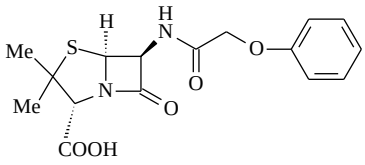


Beta-lactam Antibiotic - 7

$C_{17}H_{18}O_6N_2S$

Aspirin (0.316g, 2mmol), triethylamine (0.20 mL, 2mmol) was dissolved in dry DMF (2.5 mL). The reaction mixture is cooled to -10ºC in ice/salt bath and ethyl chloroformate (0.217 mL, 2 mmol) was added drop wise to the reaction mixture was left to stir for 20 min followed by the addition of dry ice cold acetone (2.5 mL).
A chilled solution was prepared previous day containing 6-APA (0.433g, 2mmol), triethylamine (0.20 mL, 2 mmol) in water (5 mL) was added and the reaction is left to stir for 90 min and is allowed to return to room temperature [12]. The reaction mixture is worked up as in procedure 2.2.1.

2.2.6 Preparation of Phenoxyacetic acid to 6-Aminopenicillanic acid

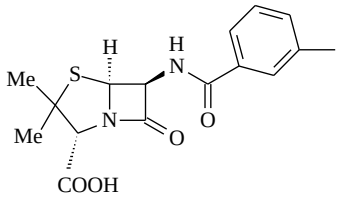


Beta-lactam Antibiotic - 12

$C_{16}H_{18}O_5N_2S$

Phenoxyacetic acid (0.304 g, 2 mmol), triethylamine (0.20 mL, 2 mmol) was dissolved in dry DMF (2.5 mL). The reaction mixture is cooled to -10ºC in an ice/salt bath and ethyl chloroformate (0.217 mL, 2 mmol) was added drop wise to the reaction mixture was left to stir for 20 min followed by the addition of dry ice cold acetone (2.5 mL). A chilled solution was prepared previous day containing 6-APA (0.433 g, 2 mmol), triethylamine (0.20 mL, 2 mmol) in water (5 mL) was added and the reaction is left to stir for 90 min and is allowed to return to room temperature. The reaction mixture is worked up as in procedure 2.2.1.

2.2.7 Preparation of m-Toluic acid to 6-Aminopenicillanic acid

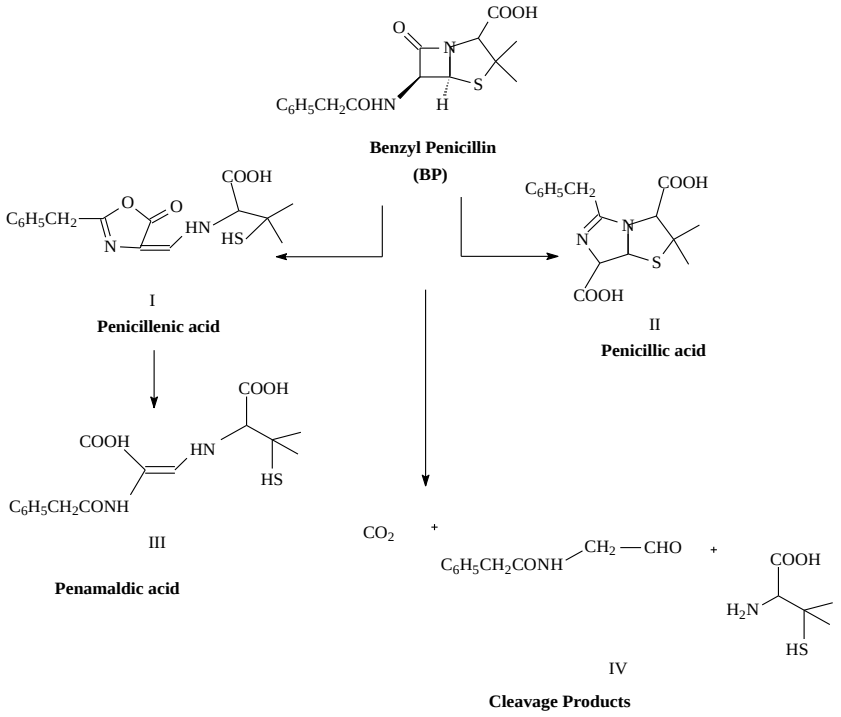


Beta-lactam Antibiotic - 14

$C_{16}H_{18}O_5N_2S$

M-Toluic acid (0.272 g, 2 mmol), triethylamine (0.20 mL, 2 mmol) was dissolved in dry DMF (2.5 mL). The reaction mixture is cooled to -10ºC in ice/salt bath and iso-butyl chloroformate (0.273 mL, 2 mmol) was added drop wise to the reaction mixture was left to stir for 20 min followed by the addition of dry ice cold acetone (2.5 mL). A chilled solution was prepared previous day containing 6-APA (0.433 g, 2 mmol), triethylamine (0.20 mL, 2 mmol) in water (5ml) was added and stirred for 90 min and is allowed to return to room temperature. The reaction mixture is worked up as in procedure 2.2.1.

2.2.8 Hydrolysis of Benzyl Penicillin and β-lactam Antibiotic 12



β-lactam drugs undergo acid-catalyzed hydrolysis when administered orally which inactivates the antibiotics. The hydrolysis pathway was explicated by scanning the antibiotic samples using UV-Vis Spectrophotometer. A stock solution of concentration $8x10^{-4}$ is prepared using Benzyl penicillin as a reference and pure moieties of synthesized β-lactam antibiotic derivative (β-lactam Antibiotic 12) and us diluted with 0.2 mol/dm^3 of HCl stock solution made up of concentrated Hydrochloric acid. Water is kept as a standard such that the instrument is balanced with the water sample. The samples are diluted to 1:1 ratio; the quartz cuvettes were washed and clean dried and to make sure that it doesn't create any imperfection in the optical path. Penicillin is dissolved in water, of antibiotic (1.5 mL) & HCl (1.5 mL) is added to check the absorbance. Care should be taken that as soon as the HCl is added the solution should be mixed using a pipette and the scanning should be done immediately. Three separate runs are made in different wavelengths at 322nm, 290nm and 240nm to record the rate of dissociation [10].

a. Calculation for Benzyl Penicillin at 240nm

Slope (K) = -0.0041
4.10 E – 03
Total A=II + III
A= 0.977
AIII = 0.271
AII = 0.706
Moles II = 0.000108615
Mole fraction = 0.271538462
% Yield of the Final Product II = 27%
% Yield of the Final Product III = 4%
% Yield of the Final Product IV = 69%

b. Calculation for Benzyl Penicillin at 290nm

Slope (K) = -0.0043
Slope = - 4.30E-03
AIII = 0.271
Mole Absorbtivity = 15800
Moles = 1.71519E-05
Moles fraction = 0.042879747
% Yield of the Final Product III = 4%

2.3 Preparation of nutrient agar media for bacterial culture

Accurately Peptone (2g), Beef extract (1.2g), Sodium Chloride (2g), Agar agar (7g) was weighed and made up to a solution by adding Distilled water (400 mL). The pH was adjusted to 7.2 and transferred all the contents into the conical flask and plug with cotton. Media was autoclaved at 121°C for 15min at 15 lbs pressure. The sterilized media was used for the preparation of agar media plates.

2.4 Anti-bacterial assay of β-lactam moieties on *α-Hemolytic Streptococcus* bacteria

The media plates prepared was swabbed with the dilute solution of *α-Hemolytic Streptococcus* bacteria. Standard antibiotic discs like Streptomycin, Ampicillin, Penicillin and Tetracycline (10µg conc.) obtained from Hi-Media; Bangalore was inoculated into the media to compare as a standard. The synthetic antibiotics were diluted with the concentration of 100 mg/mL of methanol. Pre-sterilized discs (Whatman No.42) were used as the synthetic drug antibiotic discs. The discs treated with the antibiotics were placed on the media plates swabbed with *α-Hemolytic Streptococcus* bacteria. The antibiotic treated media plates were incubated at 37°C for 24hrs. The zone of inhibition was measured.

3 Results

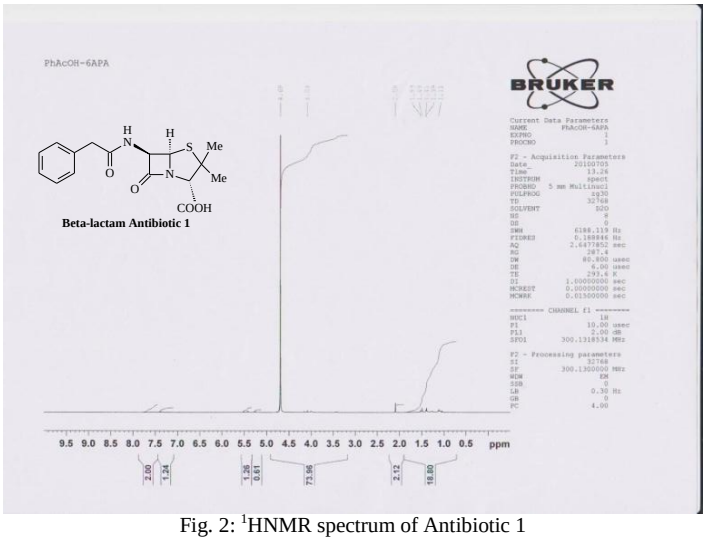


Fig. 2: [1]HNMR spectrum of Antibiotic 1

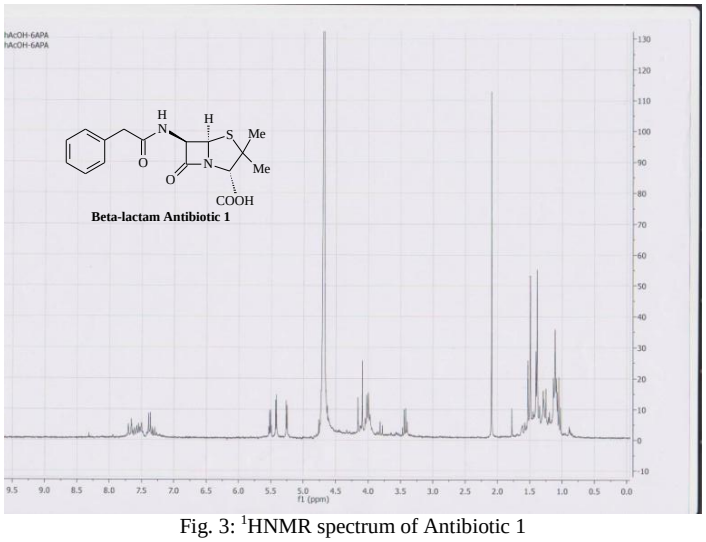


Fig. 3: [1]HNMR spectrum of Antibiotic 1

^{1}H NMR (300MHz, D_2O): δ_H 1.53 (s, 6H, CH_3), 2.09 (s, 2H, CH_2), 4.7 (s, 1H, β-lactam H) 5.25 (d, 1H, β-lactam H), 5.45 (d, 1H, β-lactam H), 8.05 (s, 1H, H)

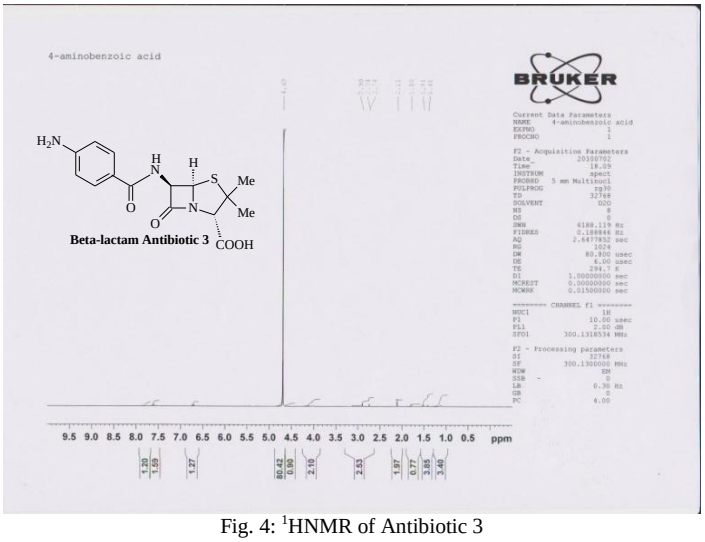


Fig. 4: ^{1}HNMR of Antibiotic 3

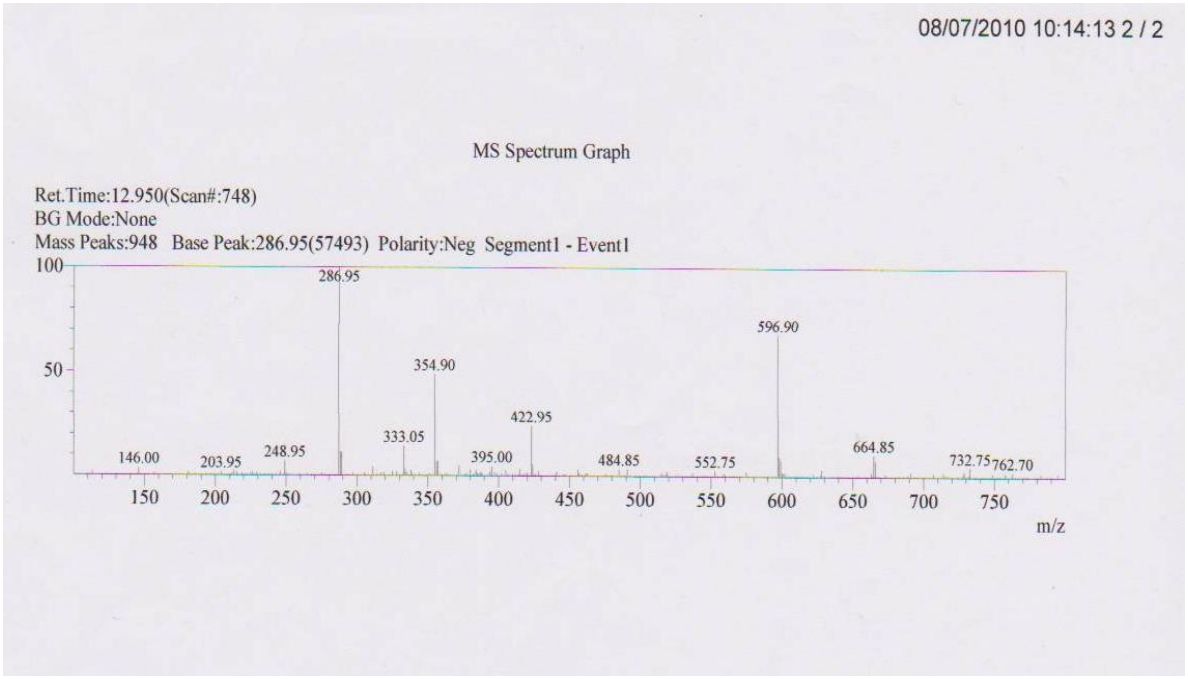


Fig. 5: Mass Spectrum of Antibiotic 3

^{1}H NMR (300MHz, D_2O): δ_H 1.40 (s, 3H, CH_3), 1.51 (s, 6H, 2xCH_3), 4.13 (s, 2H, CH_2), 5.25 (d, 2H, H), 5.45 (d, 2H, H) 7.85 (s, NH, 1H)

IR: vcm^{-1} 3232.6 (m, N-H Stretch), 1602.4 (s, C=O Stretch), 1060.4 (s, C-O Stretch), 1766.4(s, β-lactam Stretch) cm^{-1}
LC-MS (ES$^-$): Calculated mass-287.077 formula ($C_{11}H_{15}N_2O_5S$) m/z 286.95 (100%, M-H) $^-$

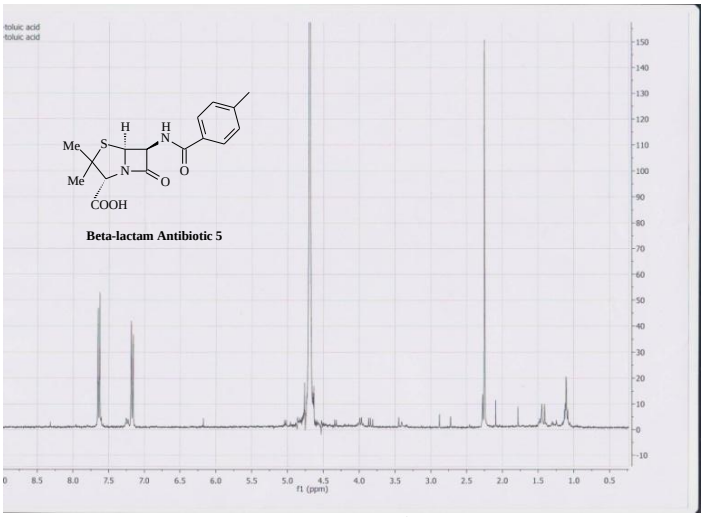


Fig. 4: NMR Spectrum of Antibiotic 5

^{1}H NMR (300MHz, D_2O): δ_H 1.55 (s, 3H, CH_3), 2.26 (s, 3H, CH_3), 4.90 (d, 1H, β lactam-H), 5.20 (d, 1H, β lactam-H) 7.63-7.66 (m, 4H, ArH)

IR: vcm^{-1}: 3371.327 (m, N-H Stretch), 1673.921 (s, C=O Stretch), 2978.992 (m, C-H Stretch) 1770.493 (s, β-lactam Stretch) cm^{-1}

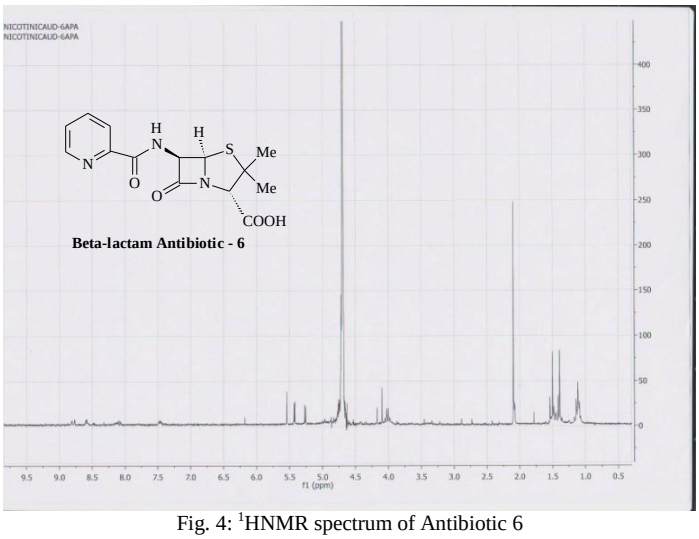


Fig. 4: ^{1}HNMR spectrum of Antibiotic 6

^{1}H NMR (300MHz, D_2O): δ_H 1.50-1.65 (m, 4H), 2.10 (s, 6H, CH_3), 4.10 (d, 1H, β lactam-H), 5.25 (d, 1H, β lactam-H), 5.45 (d, 1H, β lactam-H)

IR: vcm^{-1} 3321.112 (m, N-H Stretch), 1674.005 (s, C=O Stretch), 2970.549 (m, C-H Stretch), 1770.418 (s, β-lactam Stretch) cm^{-1}

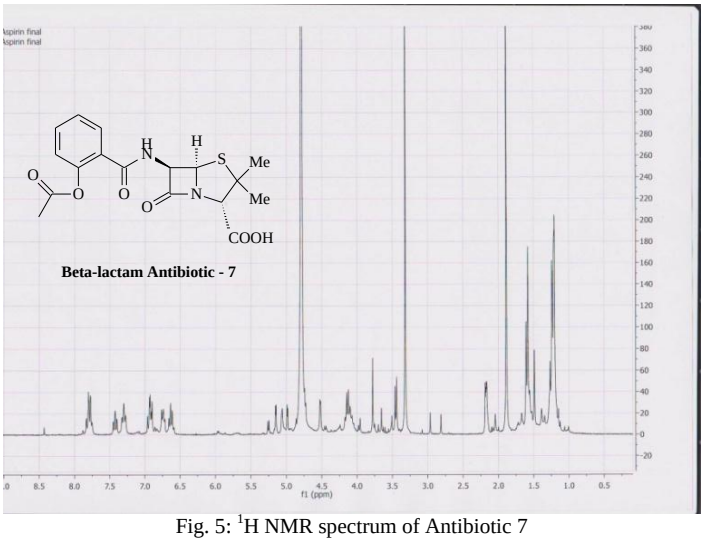


Fig. 5: ^{1}H NMR spectrum of Antibiotic 7

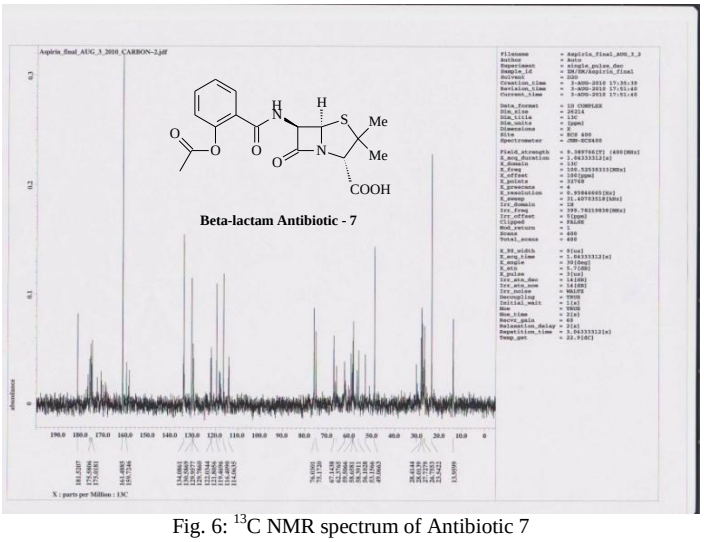


Fig. 6: ^{13}C NMR spectrum of Antibiotic 7

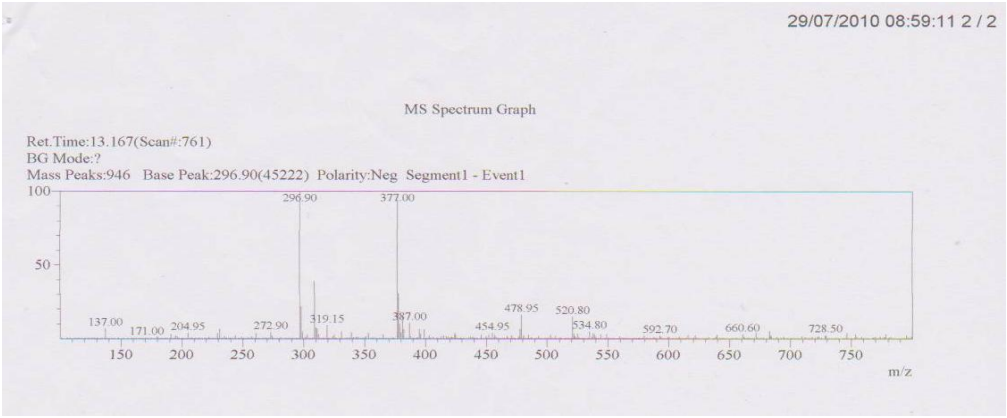


Fig. 7: Mass spectrum of Antibiotic 7

^{1}H NMR (300MHz, D_2O): δ_H 2.10 (s, 6H, CH_3), 4.10 (d, 1H, β lactam-H), 5.25 (d, 1H, β lactam-H), 5.45 (d, 1H, β lactam-H), 6.51-7.71 (m, 4H, ArH).

^{13}C NMR (75MHz, D_2O): δ_C 23.5422 ($C^{21}$and C^{22}), 13.9599 (C^{1}), 122.0344 (C^{7}), 181.5207 (C^{15}), 175.5806 (C^{11}), 76.0301 (C^{18}), 75.1720 (C^{16}), 67.1438(C^{19}), 144.0635-134.0861 (C^{5}- C^{9}).

IR: vcm^{-1}: 3282.723 (m, N-H Stretch), 1581.230 (s, C=O Stretch), 2965.211 (m, C-H Stretch), 1336.747 (s, C-O Stretch) cm^{-1}

LC-MS (ES^-): Calculated mass – 378.0885, molecular formula ($C_{17}H_{18}N_2O_6S$) m/z 377.00 (100%, M-H)$^-$.

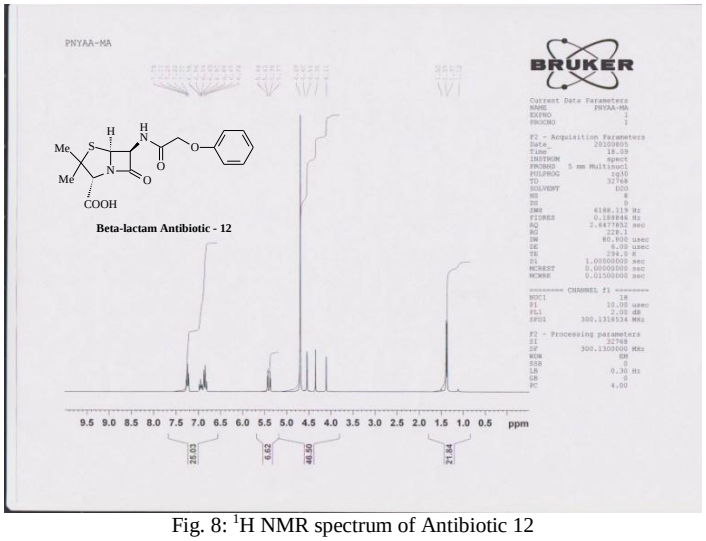


Fig. 8: ^{1}H NMR spectrum of Antibiotic 12

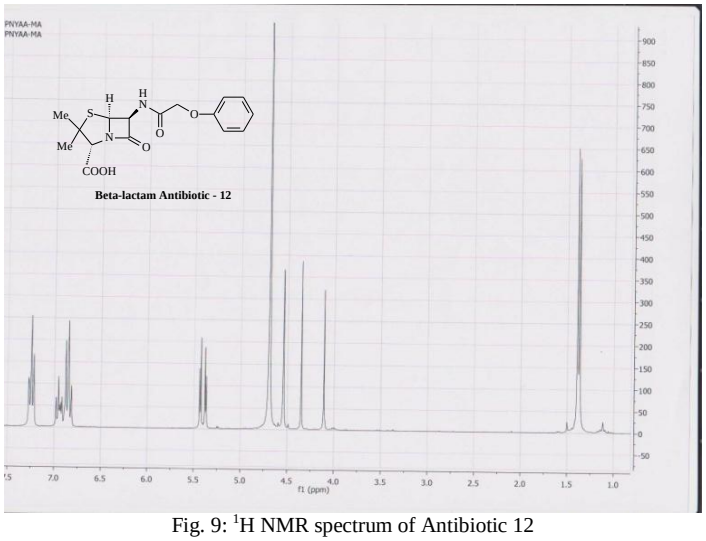


Fig. 9: ^{1}H NMR spectrum of Antibiotic 12

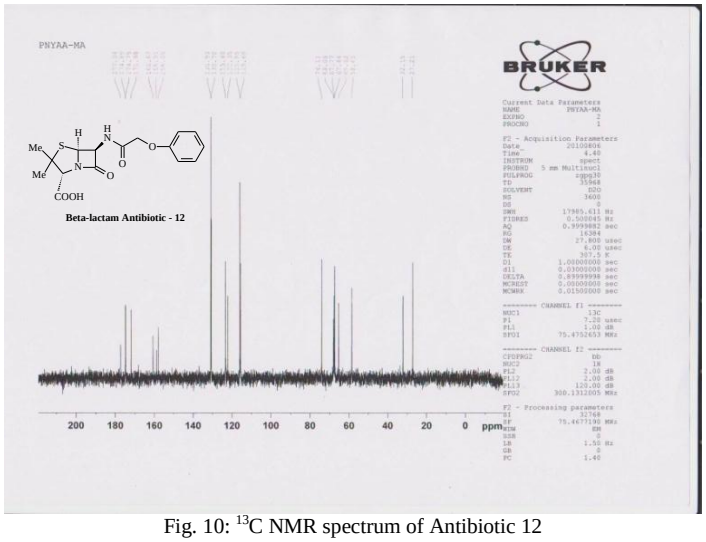


Fig. 10: ^{13}C NMR spectrum of Antibiotic 12

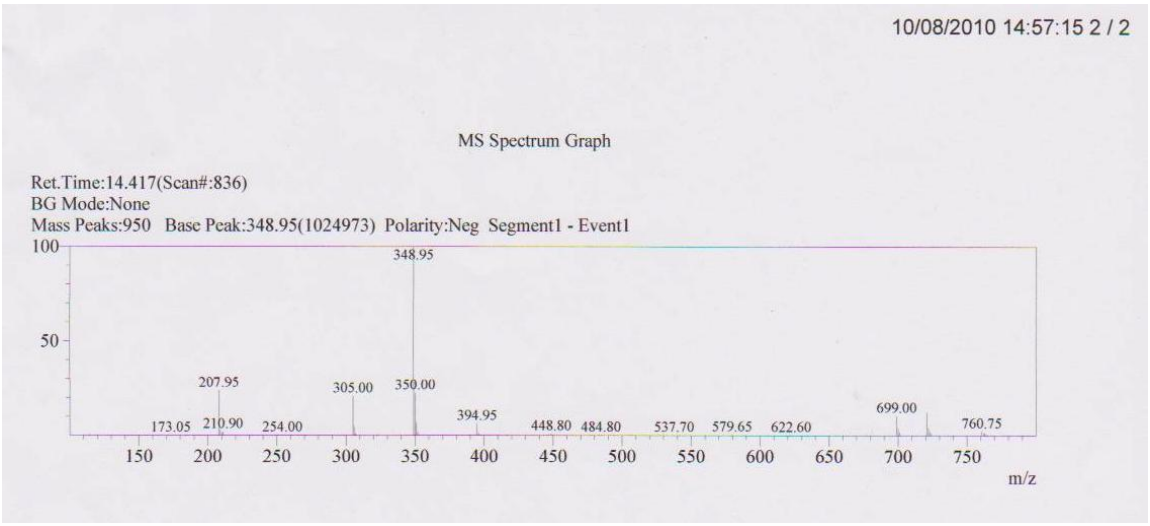


Fig. 11: Mass spectrum of Antibiotic 12

^{1}H NMR (300MHz, D_2O): δ_H 1.37 (s, 6H, β-lactam CH_3), 4.11 (s, 1H, β-lactam H), 4.35 (s, 2H CH_2)4.60 (s, 1H, β lactam-H), 5.38 (d, 1H, β lactam-H), 6.82-7.28 (m, 5H, ArH)

^{13}C NMR (75MHz, D_2O): δ_C 27.21 ($C^{19}$and C^{20}), 59.65 (C^{12}), 65.42 (C^{17}), 67.44 (C^{8}), 68.08 (C^{14}), 74.11 (C^{16}), 122.30 (C^{4}), 130.70 (C^{5} and C^{3}), 158.91 (C^{1}), 171.98 (C^{9}), 174.75 (C^{13}) 74.11 (C^{16})

IR: vcm^{-1}: 3370.447 (m, N-H Stretch), 1607.292 (s, C=O Stretch), 2965.651 (m, C-H Stretch), 1048.806 (s, C-O Stretch), 1769.903 (s, β-lactam Stretch) cm^{-1}

LC-MS (ES^-): Calculated mass - 350.093, molecular formula ($C_{16}H_{18}N_2O_5S$) m/z 348.95 (100%, M-H).

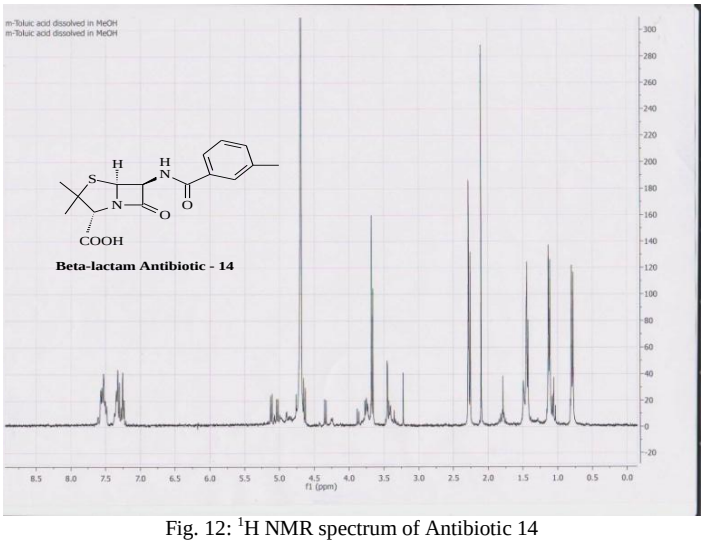


Fig. 12: ^{1}H NMR spectrum of Antibiotic 14

^{1}H NMR (300MHz, D_2O): δ_H 1.75 (s, 6H, β-lactam CH_3), 2.26 (s, 3H, CH_3), 5.10 (d, 1H, β-lactam H), 5.25 (d, 1H, β lactam-H), 7.20-7.70 (m, 4H, ArH)

IR: vcm^{-1}: 3322.035 (m, N-H Stretch), 1626.416 (s, C=O Stretch), 2929.366 (m, C-H Stretch), 1770.196 (s, β-lactam Stretch) cm^{-1}. LC-MS (ES^-): Calculated mass – 334.0984, molecular formula ($C_{16}H_{18}N_2O_4S$) m/z 333.00 (100%, M-H)$^-$

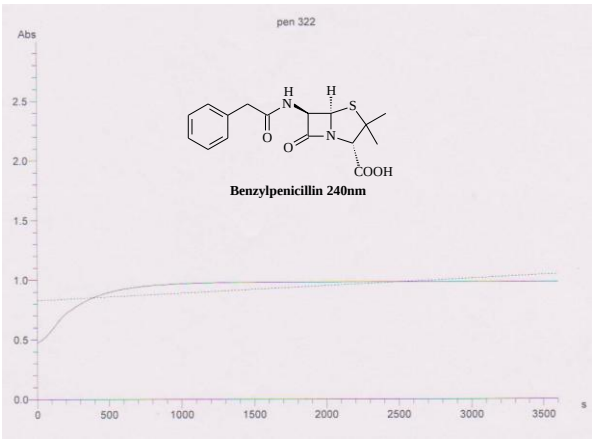


Fig. 13: Hydrolysis of benzyl penicillin at 240nm

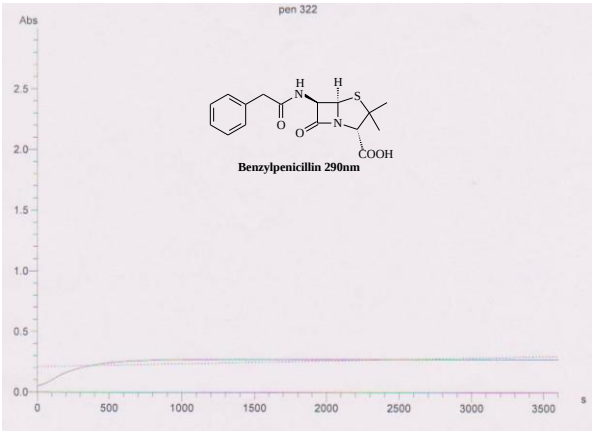


Fig. 14: Hydrolysis of benzyl penicillin at 290nm

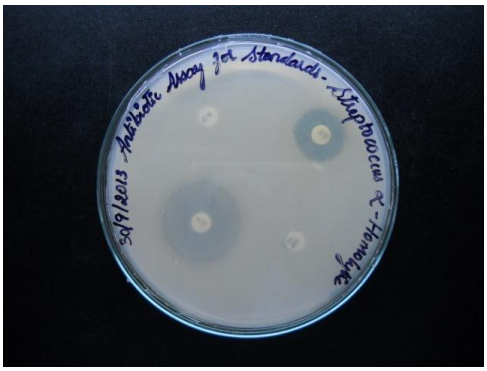


Fig. 10: Assay for Streptomycin, Ampicillin, Penicillin and Tetracycline

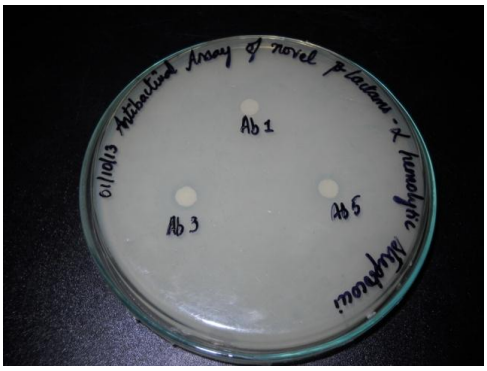


Fig. 11: Assay for antibiotic 1, 3 & 5

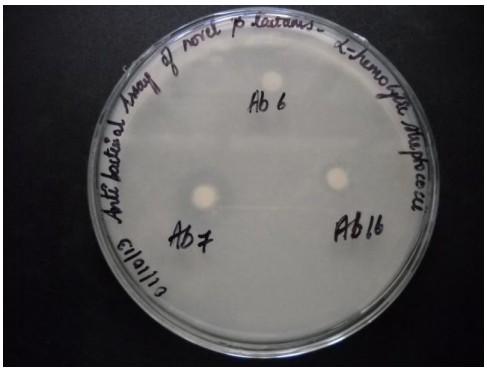


Fig. 12: Assay for antibiotic 6, 7 & 16

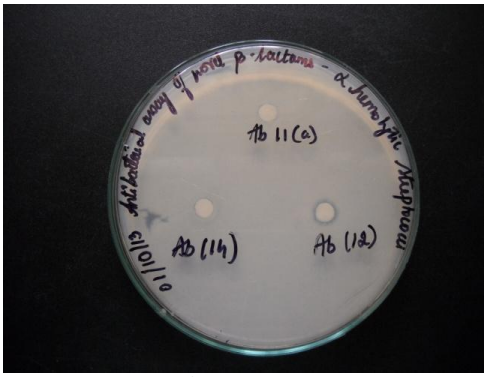


Fig. 13: Assay for antibiotic 11(a), 14 & 12

Table 2: Anti-bacterial assay of β-Lactam drugs on α-Hemolytic Streptococcus

Antibiotics	Zone of Inhibition (cms)
Streptomycin	3.0
Tetracycline	2.0
Ampicillin	0.0
Penicillin	0.0
Antibiotic 1	0.3
Antibiotic 3	0.3
Antibiotic 5	0.2
Antibiotic 6	0.3
Antibiotic 7	0.9
Antibiotic 16	0.5
Antibiotic 12	0.4
Antibiotic 11(a)	0.3
Antibiotic 14	0.3

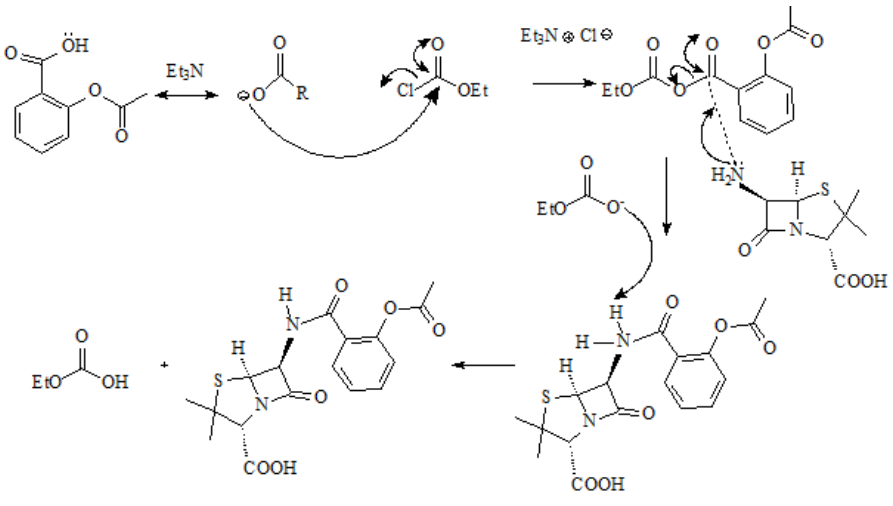
4 Discussion

Phenylacetic 6 Amino Penicillanic acid was synthesized using mixed anhydride method using ethylchloroformate in presence of triethylamine as a base gave a yellow colored solution which was further worked up to get the Potassium salt of Penicillin [8]. As the penicillin is dissolved in methanol, product obtained was dissolved in Methanol and Ether which precipitates out the inorganic salt was discarded and the dissolved mixture was subjected to evaporation under vacuum pressure. The obtained product was hygroscopic that it would turn to oil when exposed to air. The obtained product was dried using dry Acetone or Petrol 40-60. The obtained product was crystal pale yellow solid. A characteristic doublet peaks at δ 5.25 and 5.45ppm of β-lactam protons was observed in the ^{1}H NMR confirming the formation of the β-lactam protons in the product obtained.

Due to the successful coupling of 6-APA with phenylacetic acid, the further synthesis was focused on aminobenzoic acids, 4 aminobenzoic acid was used to couple with 6 APA which resulted in the ethoxy penicillin (β-lactam Antibiotic 3) because the ethylchloroformate is deactivated and forms the ethoxy penicillin.

Synthesis of β-lactam Antibiotic 5 (p-Toluic 6 amino penicillanic acid) by coupling of p-Toluic acid to 6 APA via mixed anhydride resulted in a total yield of 40%. A characteristic doublet peaks was observed at δ 4.90ppm and 5.20ppm for the β-lactam-proton moieties respectively. A medium N-H Stretch was observed in 3371.327 cm^{-1} and a strong β-lactam stretch at 1770.493 cm^{-1} was observed with respect to IR spectrum.

With the satisfactory results with (β-lactam Antibiotic 6), further synthesis was focused on the carboxylic acids with the pyridine ring in a 2 mmol scale. Nicotinic acid and 6 APA was coupled via mixed anhydride, where the results were quite promising. A characteristic doublet peaks was noted at δ 4.10, 5.25 and 5.45ppm holding the β-lactam proton peaks. A medium stretch at was noted at 3321.112 cm^{-1} which gives a promising amide stretch with respect to IR spectrum.



Scheme. 2: Proposed reaction mechanism for β-lactam antibiotic 7

Further synthesis was to couple the drugs with antipyretic and antibiotic moieties. (β-lactam Antibiotic 7) was the novel product as it was identified by the database reaxys and scifinder. Acetyl salicylic acid an antipyretic drug and antiplatelet drug which is used in cardiovascular treatment was coupled with 6-APA gave an excellent result with a yield of 70%. A characteristic peak was observed in ^{1}H NMR; doublets peaks at δ 4.10, 5.25 and 5.45ppm assigning β-lactam protons and an ArH multiplets peak at δ 6.51-7.71. ^{13}C NMR was gave a confirmation of the carbon atoms of β-lactam ring at 75.1720 (C^{16}) and thiazolidine ring at 67.1438 (C^{19}). LC-MS of the compound confirmed the formation of the Acetyl salicylic penicillin and gave $[M-H]^{-}$ peak at 377.00 m/z calculated molecular weight from exact isotopic masses being 378.0885 g/mol.

Phenoxyacetic acid was a novel compound as it was identified by the database reaxys and scifinder chosen as the acid to couple with 6-APA to form a phenoxy-6-APA derivative because the positioning of the acid carbonyl group. It is not directly adjacent to the aromatic ring and would therefore doesn't form ethoxy penicillins.

The (β-lactam Antibiotic 12) was synthesised as a fine white crystalline solid yielding 75 % of the product. The ^{1}H-NMR exhibit two doublets at δ 4.60, and 5.38 ppm that is characteristic of the β-lactam protons. ^{13}C displayed δ_C 27.21 ($C^{19}$and C^{20}) of CH_3 groups and 74.11 (C^{16}) carbon of the thiazolidine ring, 59.65 (C^{12}), 68.08 (C^{14}), 174.75 (C^{13}) carbons of β-lactam ring. LC-MS of the compound gave a peak at m/z 348.95 $[M-H]^{-}$ calculated mass being 350.093 g / mol.

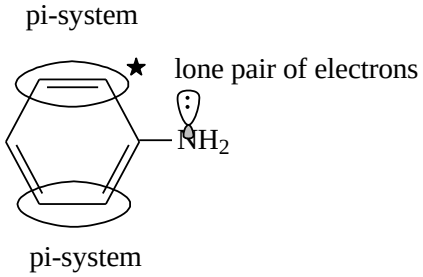
Further synthesis was to couple m-Toluic acid to 6 APA using iso-butylchloroformate. (β-lactam Antibiotic 14) was the novel product as it was identified by the database reaxys and scifinder. M-Toluic acid was coupled with 6-APA gave an excellent result with a yield of 20%. A characteristic peak was observed in ^{1}H NMR; doublets peaks at δ 5.10, 5.25ppm assigning β-lactam protons and a multiplets peak of 4 aromatic protons at δ 7.20-7.70. LC-MS of the compound confirmed the formation of the m-Toluic penicillin moieties and gave $[M-H]^{-}$ peak at 333.00 m/z calculated molecular weight from exact isotopic masses being 334.0984 g/mol.

Further synthesis was focused to couple Phenylacetic acid to 6 APA using iso-butylchloroformate. This (β-lactam Antibiotic 16) Phenylacetic acid was coupled with 6-APA gave an excellent result with a yield of 30%. Isolation of Penicillin was unsuccessful, ^{1}HNMR revealed the isolation of starting material.

NHS/DCC Method: Reactions were initially carried out at room temperature with Phenylacetic N-hydroxysuccinimide ester was coupled with 4-tert-butylaniline and 4-Chloroaniline to form Compound 1 and Compound 7 with yields of 50% and 53% respectively. A modification by heating the reaction mixture at 100ºC for 2 h gave an amide product [10]. The formation of the amide in Compound 1 was confirmed by the distinctive broad singlet peak in the ^{1}H NMR at δ 6.99 ppm. IR and LC-MS was consequently used to fully exemplify the amide. The formation of the amide in Compound 7 was confirmed by the distinctive broad singlet peak in the ^{1}H NMR at δ 7.10 ppm. IR and LC-MS was consequently used to fully exemplify the amide. Substituent on the side ring of anilines has no apparent effect.

The same method replacing acids as 2-Iodobenzoic acid and 3-Chlorobenzoic acid was coupled to form Compound 2 and Compound 4 with equal yield of 18%. Amide product obtained by this method resulted in a less yield it was then followed by flash chromatography. The amine/anilines don't appear to have an effect.

This problem is due to the presence of chlorine molecules in 2nd and 6th position of the aromatic ring. The lone pair of electrons on the Anilines conjugates the π-system of the aromatic ring and makes the π- system electron rich. The chlorine on the aromatic ring pulls the electrons of p-orbitals out of the π-system and makes π-system electron deficient. And therefore the lone pair of electrons of the nitrogen fails to attack the carbonyl carbon.



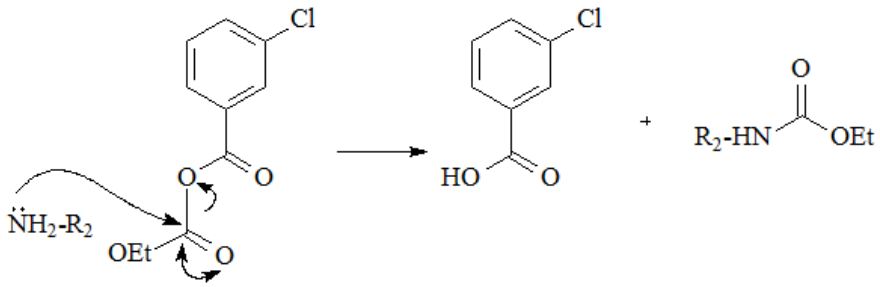
The ability of reactivity of the anilines in a sequence of the reaction was difficult and that this was mainly due to the reduced nucleophilicity of the aniline nitrogen. The poor reactivity of the anilines is due to the increased conjugation of the lone pairs present on the nitrogen molecule with the π system of the aromatic ring. This was driven by the increased electron deficiency caused by the electron withdrawal by the chlorine substituents on the aromatic ring.

Preparation of compound 2 and 4 proved to be successful and carrying out the reactions overnight resulted in the formation of the amides were confirmed by ^{1}H-NMR that resulted in the characteristic broad singlet peaks at δ 5.39 ppm and δ 6.59 ppm respectively.

Mixed Anhydride method: Another pilot method scrutinized was activation of the chosen acid by the formation anhydride by coupling with ethyl chloroformate followed by direct addition of the amine/aniline to give the resulting amide (Scheme. 2). Attack by the anilines or amines take place at the acid carbonyl with loss of the ethoxy carboxylate.

This was due to electron donation to the chloroformate carbonyl by both of its oxygen substituents. Thereby stabilising the carbonyl carbon and reducing its electrophilicity and directing the attack at the highly reactive carbonyl group of an acid [9].

Compounds 3, 5 and 6 were prepared using the mixed anhydride method by the activation of the acid by ethyl chloroformate. Compounds 3, 5 and 6 were produced in 53%, 16% and 4% respectively. In the ^{1}H-NMR confirming the formation of the amide in the above mentioned compounds show a characteristic broad singlet peak 4.99 ppm (Compound 3), 6.58 ppm (Compound 5) and 6.58 ppm (Compound 6).



Scheme. 3: Proposed Mechanism for the formation of Ethoxy amides using Ethylchloroformate in Mixed Anhydride method

Formation of the ethoxy amide is the reduced reactivity of the acid carbonyl by increased conjugation with the adjacent π system of the aromatic ring. The chlorine molecule in the *ortho* position of the chosen aromatic acid has an electron withdrawing effect on the ring increasing the electron deficiency of the aromatic system. This increases the conjugation between the aromatic ring and the directly attached carbonyl reducing its reactivity and susceptibility to nucleophilic attack from the aniline/amides. The chlorine substituent on the aromatic acid may also provide some steric hindrance further reducing the vulnerability of the carboxylic acid carbonyl to nucleophilic attack. Due to this feature to decreases the difference in reactivity of the carbonyl carbon of the aromatic acid and the ethoxy carbonyl carbon that allows attack leading the ethoxy amide as a major product.

Although the formation of the ethoxy amide, it was made certain that the mixed anhydride method was the most suited for the coupling of aromatic acids with 6-APA compared to the DCC / NHS method. Mixed anhydride method was selected to synthesize the β-lactam derivatives as it was proved by the model amide reactions.

The antibacterial assay on α-Hemolytic Streptococcus gives the better zone of inhibition with Antibiotics 7, 12 and 16. The bioassay was also carried out with the *Staphylococcus aureus* but the β lactam antibiotics fails to inhibit the growth of bacteria.

5 Summary

There is eternal research on the discovery of novel antibiotic to overcome the antibiotic resistant strains of bacteria like Mycobacterium tuberculae. Objective of my research is to develop the pharmacodynamic property of the β-lactam drugs. The novel β-lactam antibiotic moieties which are synthesized were mainly via the mixed anhydride method using ethyl chloroformate or iso-butylchloroformate. A novel compounds (β-lactam antibiotic 7), (β-lactam antibiotic12), (β-lactam antibiotic14) moieties exhibit good result with ethylchloroformate and iso-butyl chloroformate with a good yield. Which can be further studies need to be performed in this area of research by using techniques such as reverse phase HPLC to provide quantitative results.

Another novel compound β-lactam antibiotic 14 worked well with iso-butylchloroformate because the iso-butylchloroformate creates a steric hindrance to the carbonyl carbon and paves the way for nucleophilic attack on the carbonyl carbon of the chosen aromatic acid and thereby inhibiting the ethoxy contaminants.

DCC method was carried out using NHS as an activating reagent for the carboxylic acids, it fails to couple with amines/anilines due to the ability of reactivity of the anilines was difficult sequence of the reaction and this was mainly due to the reduced nucleophilicity of the aniline nitrogen. The poor reactivity of anilines is due to the increased conjugation of the lone pair of electrons present on the nitrogen molecule with the π-system of the aromatic ring. This was driven by the increased electron deficiency cause due to the electron withdrawal by the chlorine substituents on the aromatic ring.

But when DCC method was carried out in presence of DMAP as a nucleophilic catalyst, DMAP makes 6-APA more nucleophilic and thereby increasing the coupling activity. I conclude that the method used for the synthesis of penicillin antibiotics can be carried out with different moieties of chloroformate to get better and purified β-lactam derivatives.

The bioassay reveals that the synthesized antibiotics show antibacterial activity on *α-Hemolytic Streptococcus*. Antibiotic 7, 12 and 16 shows the maximum zone of inhibition in the assay (Fig. 12 and 13). β-lactam ring acts as a highly effective acylating agent due to its particularly strong electrophilic reactivity. Thus, it is now known that the synthesized antibiotics irreversibly acylates the bacterial transpeptidase enzyme responsible for the cross-linking reaction which unites the terminal glycine residue of a pentaglycine strand with the D-ala residue of a neighbouring pentapeptide, in a crucial step during the construction of bacterial cell walls. The acylation process deactivates this crosslinking enzyme, thereby compromising the integrity of the bacterial cell wall, resulting in rapid cell death.

Acknowledgements

Authors would like to thank Dr. Michael J Hall, Newcastle University, for his continuous assistance and we also acknowledge Dr. Zuleyka McMillan for helping in NMR and UV-Vis spectral analysis.
Special thanks to NewChem Technologies faculty members, Dr Kristin Downey, Mrs. Anitha.Kasa.Prahlad for LC-MS analysis of drug samples and Dr. Rachel Campbell for helping with experimental procedures. Many thanks to our lab demonstrator Dr. Carl Young and Dr. Pedro Villuendas for analyzing the spectral results. Authors thank lab technicians James, Helen for their continuous help and support in the Bedson Teaching Laboratory.
Authors heartily thank Dr. B N Ramdev (HOD), Dr. Sujaya (Assistant Professor) Department of Medical Physiology; Dr. H.P. Pundarikaksha (HOD), Dr. K. Girish (Professor), Dr. B.R. Kanchana (Associate Professor), Dr. K. Vasundara Krishnaiah (Associate Professor) and Dr. R. Jyothi (Associate Professor) Department of Medical Pharmacology, Kempegowda Institue of Medical Sciences, Bangalore (India).
Authors specially thank Gauthama Reddy for procuring bacterial cultures for the bioassay studies.

References

[1] Fischbach, M.A., Walsh. C.T., Antibiotics of Emerging Pathogens, Science, 2009, Vol.325, p 1089-1093.
[2] An Introduction to Medicinal Chemistry (2nd Edition) by Graham L. Patrick.
[3] www.scripps.edu/chem/nicalaou/images/sample chapter13.pdf cited on 20/04/2010.
[4] Donald J. Tipper and Jack L. Strominger, Mechanism of action of Penicillins: A proposal based on their structural similarity to Acyl-D-alanyl-D-alanine, 1965 Vol.54, No.4, pp 1133-1141.
[5] www.mikeblaber.org/oldwine/BCH4053/lecture15/lecture15/html.
[6] G.N Rolinson, A.M. Geddes, The 50th anniversary of the discovery of 6-aminopenicillanic acid (6-APA), International journal of Antimicrobial agents Vol. 29 (2007) p.3-8.
[7] Penicillin project for MSc students; Newcastle University.
[8] Bertil Ekstrom; Gomez-Rivella, A; Mullberg. R; Thelin H; Sjoberg B., Semisynthetic Penicillins, Acta Chem.Scand. 1965, Vol. 19, p. 281 - 299.
[9] W.Clark. Still, Michael Kahn and Abhijit Mitra, Rapid chromatographic technique for preparative separations with moderate resolution. J.Org.Chem, Vol 43, No. 14, 1978.
[10] McCarrick, T.A; McLafferty, F.W., Penicillin Hydrolysis: a kinetic study of multistep, multiproduct reaction, J.Chem.Educ 1984, 61, 463-464.